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(54) Title: MEASUREMENT OF ANALYTE CONCENTRATION

(57) Abstract

The concentration of an analyte, such as a hormone or other biologically active material, in a fluid, especially a body fluid, is measured by contacting the fluid with a trace amount of a binding agent, such as an antibody, specific for the analyte, determining a figure representative of the proportional occupancy of binding sites on the binding agent and estimating from that figure the analyte concentration. Provided that the amount of binding agent is sufficiently small that its introduction has no significant effect on the total free analyte concentration, the proportional occupancy of binding sites is independent of the volume of the fluid and hence it is not necessary to measure accurately beforehand the volume of the fluid or fluid sample being tested. It is therefore possible to design a concentration-measuring device for insertion into a body fluid of a living creature for *in situ* measurement of concentration.

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Measurement of analyte concentration

Technical Field

The present invention relates to the measurement of ambient analyte concentrations in fluids, primarily the concentrations of hormones and other biologically active substances in body fluids such as saliva, serum, blood and urine.

Background Art

It is known to measure the concentration of hormones in body fluids by contacting a fluid sample of accurately determined volume with a binding agent having binding sites specific for the hormone, usually an antibody, and radioactively labelled hormone. The binding agent binds a proportion of the unlabelled hormone and a proportion of the labelled hormone, the relative amount of labelled hormone bound being a function of the amount of unlabelled hormone present in the sample. The results obtained are calibrated by comparison with the results obtained with standard solutions containing known concentrations of unlabelled hormone, and thus the actual amount of unlabelled hormone in the unknown sample is determined.

Important disadvantages of the known technique are that a sample of the body fluid in question has to be removed from the body and placed in a test tube or the like and its absolute volume needs to be known. It would be an advantage to avoid one or both of these difficulties.

Disclosure of Invention

According to the invention means are provided whereby the ambient concentration of an analyte, such as a hormone, in a fluid can be measured without the need to know the volume of the fluid being measured and hence, in the case of body fluids, without the need to remove the body fluid from the body.

This invention is based on the fact, not hitherto appreciated, that when a fluid containing an analyte such as a hormone is contacted with an antibody or other binding agent having binding sites specific for the analyte, the occupancy of the binding sites by the analyte (proportion of binding sites occupied) is independent of the absolute volume of the fluid and the absolute number of binding sites, and hence independent of the absolute amount of binding agent,

provided only that the relative amounts of analyte and binding agent and the affinity between them are such that the introduction of the binding agent into the fluid has no significant effect on the concentration of the analyte. Thus, for example, if only a trace amount of binding agent is used, such that only an insignificant fraction of the analyte becomes bound to the binding agent, then the overall analyte concentration in the fluid will not change noticeably.

Provided that the above condition holds, the concentration [H] of analyte in the fluid is related to the fraction of binding sites occupied (Ab/Ab_o) by the equation

$$\frac{Ab}{Ab_o} = \frac{K_{ab}[H]}{1 + K_{ab}[H]}$$

where K_{ab} is the equilibrium constant for the binding of the analyte to the binding sites and is a constant for the given analyte and binding agent at a given temperature.

A close analogy to this basic idea is provided by the use of a simple thermometer for the measurement of ambient temperatures. The introduction of a thermometer into - for example - a room generally implies uptake of heat by the thermometer and hence a (usually insignificant) disturbance to the pre-existing temperature of the room. Provided the thermal capacity of the room and its contents are large as compared with that of the thermometer, the temperature ultimately recorded by the thermometer essentially reflects the original room temperature. Likewise the binding-site occupancy of an antibody or other binding agent probe introduced into a biological or other fluid will, assuming the conditions mentioned above are adhered to, reflect the analyte concentration originally present in the fluid.

Accordingly it is possible to design a probe which contains immobilised binding agent in low concentrations, to insert this into the fluid whose ambient analyte concentration is to be measured and, once equilibrium has been reached, to determine the proportion of antibody sites occupied by the analyte. Such a determination will frequently be performed on the probe after withdrawal from the fluid, al-

though this is not an essential feature of the invention, and it would be advantageous in some cases to make the determination in situ and perhaps to couple it with feed-back measures to correct any imbalance of analyte concentration detected, for example to maintain a particular hormone level in a body fluid.

Therefore, the present invention provides in broad terms in one aspect a method of measuring ambient analyte concentration in a fluid, the method comprising contacting an unmeasured volume of the fluid with a trace amount of binding agent having binding sites specific for the analyte and estimating from the proportional occupancy of the binding sites the concentration of analyte in the fluid. In this context the term "trace" denotes an amount which has only an insignificant effect on the total concentration of free analyte in the fluid.

The method may be used for the estimation of analytes of all types provided that a specific binding agent is available. However, it is likely to be of greatest value in the estimation of biologically active materials such as drugs, viruses and particularly hormones, where other estimation methods are more complex. The analytes may be present in any fluid from simple aqueous solutions up to biological fluids of all types but estimation of concentrations in body fluids provides a particularly important area. The presence or absence of other ingredients is immaterial provided that they do not interfere with the binding of the analyte. The hormones may be present in fluids also containing endogenously bound hormone but this need not be the case.

A wide variety of binding agents may also be used provided that they have binding sites which are specific for the analyte in question, as compared with any other ingredient in the fluid in question. When estimating concentrations of hormones or other naturally occurring body chemicals it may be advantageous to use antibodies for the chemical in question where these are readily obtainable. However, other binding agents such as binding proteins or receptor preparations (preparations containing receptor sites and derived

from areas of the body where the chemical in question normally becomes bound) may also be used.

Conveniently, the binding agent used will be immobilised on a solid support (although soluble binding agents could be used and later precipitated or otherwise separated to enable the binding site occupancy to be estimated). The solid supports used may be those which are conventional for this purpose, incluidng cellulose, polysaccharide such as Sephadex (Registered Trade Mark) and the like. When, according to a preferred embodiment of the invention, the concentration of an analyte in a body fluid is to be estimated without removing the body fluid from the body, the support may be in any form convenient for insertion into an appropriate part of the body, for example a probe made of polystyrene or other rigid non-harmful plastics material.

Preferably the binding agent chosen will be one whose equilibrium constant K_{ab} in the above equation is such that the proportion of binding sites occupied by the analyte at its expected concentration in the fluid will be considerably less than 100%, more preferably less than 75%. This gives greater sensitivity to variations in concentration. The binding agent chosen will therefore normally be different in its thermodynamic characteristics from the antibody chosen for use in known radioimmunoassay determinations of hormone concentration, where it is desirable to have as high an occupancy of binding sites on the antibody, and hence as high an equilibrium constant, as possible.

When the occupancy of binding sites on the binding agent is to be determined by methods involving binding the unoccupied sites with a reagent whose presence is discernible, for example a radioactively labelled form of the analyte, the equilibrium constant K_{ab} is preferably such that a substantial proportion of the binding sites are occupied, advantageously at least 25%, because this gives greater sensitivity in the final measurement. Under such circumstances, the equilibrium constant of the binding agent is preferably close to the reciprocal of the expected analyte concentration because this will lead to a binding site occupancy close to 50%.

The nature of the method of estimating the occupancy of binding sites on the binding protein is not an essential part of the present invention in its broadest form and a variety of methods can be used. The simplest of these is a back-titration of unoccupied sites by the use of a labelled reagent which binds with unoccupied sites but it is also possible to use a sandwich-type or two-site approach. Alternatively, the extent of occupancy can be measured by biochemical or other means in situ.

Where back-titration assay methods are being used, it is preferred to use a binding agent whose dissociation constant for the uncoupling of analyte is low in order to avoid measurement errors as a result of premature dissociation of the analyte from the binding agent. The rate at which equilibrium is reached may also be slow, although if sufficiently small amounts of binding agent are used relative to analyte the equilibrium should be reached relatively quickly. It is also possible to make measurements before equilibrium is reached and to deduce from them the concentrations involved, but this adds to the complexity of the operation and reduces the accuracy and consequently it is not recommended.

With the use of small amounts of binding agent for the test methods it becomes of greater importance to have a labelled reagent of high specific activity for the back-titration to determine the proportion of unoccupied binding sites because, in general, only small absolute numbers of occupied and unoccupied binding sites will be present. Accordingly, instead of conventional radioisotopic labels it may be desirable to employ labels of other types such as fluorescent labels.

An added advantage of the use of fluorescent labels or others of very high specific activity for analyte-labelling is that they make possible the development of very high sensitivity, multiple-analyte, assays relying on the scanning of the distribution of fluorescent labels (comprising labelled antibodies and/or labelled analytes) deposited on the surface of - for example - a suitable plastics material.

Such a surface - "printed" with a mixture of different antibodies and subsequently exposed to the biological fluid under test - can potentially be used to reveal the concentrations of many different analytes in the same sample - a requirement which is likely to become increasingly pressing in the monitoring of blood for the presence of complex mixtures of viral antigens and/or anitbodies, tumour antigens, hormones etc.

The concept of the "immunometer" - i.e. the analyte concentration sensing device discussed in the preceding paragraph - is likely to bring about significant changes in research and of routine clinical diagnosis. For example, steroid and thyroid hormone levels may ultimately be monitored - not by analysis of blood samples as is current normal practice - but by examination of the antibody-binding site occupancy of a plastic probes following their insertion, for a few minutes, into the subject's mouth, and their exposure to ambient hormone levels present in the saliva.

The following Examples illustrate the basis for the invention

EXAMPLE 1

Antibody directed against a hormone occurring in a body fluid is diluted in a 0.05M barbital buffer at pH 8.7 and the resulting fluid is exposed to a probe in the form of a plastics support made of polystyrene for 2 to 16 hours at ambient temperature. The plastics support is then removed and thoroughly washed and can then be used as a probe to estimate the concentration of the hormone in appropriate fluids by the method of the invention after its affinity (equilibrium constant) and binding capacity have been assessed by a known method.

EXAMPLE 2.

Antibody directed against hydrocortisone (cortisol), obtained from the Tenovirus Institute for Cancer Research, Welsh National School of Medicine, Cardiff, Wales, was coupled to a solid support and the equilibrium constant (K_{ab}) and binding capacity of the resulting material were measured, by Scatchard analysis, and found to be 2×10^{10}

litres/mole and 100 pmoles/ml respectively.

Standard cortisol solutions containing cortisol concentrations of 100 pM, 1nN, 10nM, 100nM and 1 uM were prepared by dissolving pure cortisol (H4001, from Sigma Chemical Co., Poole, England) in a buffer solution of 0.05M KH_2PO_4 and 0.15M NaCl (pH 7.4) (hereinafter referred to as PBS) containing 0.1% by weight gelatine (No. 44045, from BDH Chemicals Ltd., Poole, England).

Amounts of the antibody preparation having a binding capacity of less than 10 fmoles of cortisol were incubated to equilibrium at 20°C (16 hours, although equilibrium had for practical purposes been achieved within 20 minutes) with samples of each of the standard cortisol solutions having volumes of 0.2, 0.4 and 0.8 ml. After incubation the samples were cooled, on ice, to 4°C and the solid material washed thoroughly with PBS.

The extent of occupancy of the antibody binding sites by cortisol was determined in each case by a radioimmunoassay back-titration using as the labelled material a high specific activity iodinated cortisol (~1000 Ci/m mole ^{125}I), obtained from RIA Ltd., Cardiff, Wales.

Concentrated ^{125}I-cortisol was added and mixed with the solid material in each sample and incubation was continued for 1 Hour at 4°C. The solid material was again thoroughly washed and the bound radioactivity determined. The accompanying drawing is a graph showing the relationship between the observed radioactivity (in counts per minute) and the cortisol concentration (in nM) of the samples of the standard solutions. Within the limits of experimental error, the level of bound radioactivity was the same for all three samples of identical cortisol concentration and was unaffected by their differences in volume.

CLAIMS

1. A method of measuring ambient analyte concentration in a fluid by contacting the fluid with a binding agent having binding sites specific for the analyte as compared with the other components of the fluid, determining a figure representative of the extent of binding of the analyte to the binding agent and estimating therefrom the analyte concentration in the fluid, characterised in that the binding agent is used in a trace amount which has at most an insignificant effect on the total concentration of free analyte in the fluid, and a figure representative of the proportional occupany of the binding sites on the binding agent is determined and used to estimate the analyte concentration in the fluid, whereby it is not necessary to measure accurately the volume of the fluid contacted with the binding agent.

2. A method as claimed in claim 1, characterised in that the analyte is a biologically active material and the binding agent is an antibody for the analyte.

3. A method as claimed in claim 2, characterised in that the biologically active material is a hormone, the fluid contains endogenously bound hormone as well as free hormone and the concentration of free hormone alone is estimated.

4. A method as claimed in claim 1, characterised in that the volume of the fluid contacted with the binding agent is not measured accurately.

5. A method as claimed in claim 1, characterised in that the binding agent is contacted with the fluid whilst immobilised on a solid support, and the binding agent and solid support are separated from the fluid before the figure representative of the proportional occupancy of the binding sites is determined.

6. A method as claimed in claim 1, characterised in that the fluid is a body fluid and the binding agent is immobilised on a probe capable of being inserted into and withdrawn from the body fluid.

7. A method as claimed in claim 1, characterised in that a binding agent is chosen whose equilibrium constant for the binding of the analyte to the binding sites is such that less than 75% of the binding sites of the binding agent will be occupied by the analyte at its expected concentration in the fluid.

8. A method as claimed in claim 7, characterised in that a binding agent is chosen whose equilibrium constant for the binding of the analyte to the binding sites is such that more than 25% of the binding sites of the binding agent will be occupied by the analyte at its expected concentration in the fluid.

9. A method as claimed in claim 5, characterised in that the figure representative of the proportional occupancy of the binding sites on the binding agent is determined by back-titration using a fluorescent labelled reagent.

10. A device for measuring the concentration of an analyte in a body fluid of a living creature without removing a sample of the body fluid from the living creature, characterised by a solid probe designed to be introduced into and withdrawn from the body fluid and having immobilised thereon a binding agent having binding sites specific for the analyte as compared to the other components of the body fluid, the binding agent being present in a trace amount such that the insertion of the probe into the body fluid has at most an insignificant effect on the total concentration of the free analyte in the body fluid.

AMENDED CLAIMS
(received by the International Bureau on 02 March 1984 (02.03.84)

(amended) 1. A method of measuring ambient analyte concentration in a fluid by contacting the fluid with a binding agent having binding sites specific for the analyte as compared with the other components of the fluid, determining a figure representative of the extent of binding of the analyte to the binding agent and estimating therefrom the analyte concentration in the fluid, characterised in that the binding agent is used in a trace amount which has at most an insignificant effect on the total concentration of free analyte in the fluid, and a figure representative of the proportional occupancy of the binding sites on the binding agent is determined and used to estimate the analyte concentration in the fluid, without estimating as an essential intermediate step the total amount of analyte in the fluid contacted with the binding agent, so that it is not necessary to measure accurately the volume of the fluid contacted with the binding agent.

2. A method as claimed in claim 1, characterised in that the analyte is a biologically active material and the binding agent is an antibody for the analyte.

3. A method as claimed in claim 2, characterised in that the biologically active material is a hormone, the fluid contains endogenously bound hormone as well as free hormone and the concentration of free hormone alone is estimated.

4. A method as claimed in claim 1, characterised in that the volume of the fluid contacted with the binding agent is not measured accurately.

5. A method as claimed in claim 1, characterised in that the binding agent is contacted with the fluid whilst immobilised on a solid support, and the binding agent and solid support are separated from the fluid before the figure representative of the proportional occupancy of the binding sites is determined.

6. A method as claimed in claim 1, characterised in that the fluid is a body fluid and the binding agent is immobilised on a probe capable of being inserted into and withdrawn from the body fluid.

7. A method as claimed in claim 1, characterised in that a binding agent is chosen whose equilibrium constant for the binding of the analyte to the binding sites is such that less than 75% of the binding sites of the binding agent will be occupied by the analyte at its expected concentration in the fluid.

8. A method as claimed in claim 7, characterised in that a binding agent is chosen whose equilibrium constant for the binding of the analyte to the binding sites is such that more than 25% of the binding sites of the binding agent will be occupied by the analyte at its expected concentration in the fluid.

9. A method as claimed in claim 5, characterised in that the figure representative of the proportional occupancy of the binding sites on the binding agent is determined by back-titration using a fluorescent labelled reagent.

10. A device for measuring the concentration of an analyte in a body fluid of a living creature without removing a sample of the body fluid from the living creature, characterised by a solid probe designed to be introduced into and withdrawn from the body fluid and having immobilised thereon a binding agent having binding sites specific for the analyte as compared to the other components of the body fluid, the binding agent being present in a trace amount such that the insertion of the probe into the body fluid has at most an insignificant effect on the total concentration of the free analyte in the body fluid.

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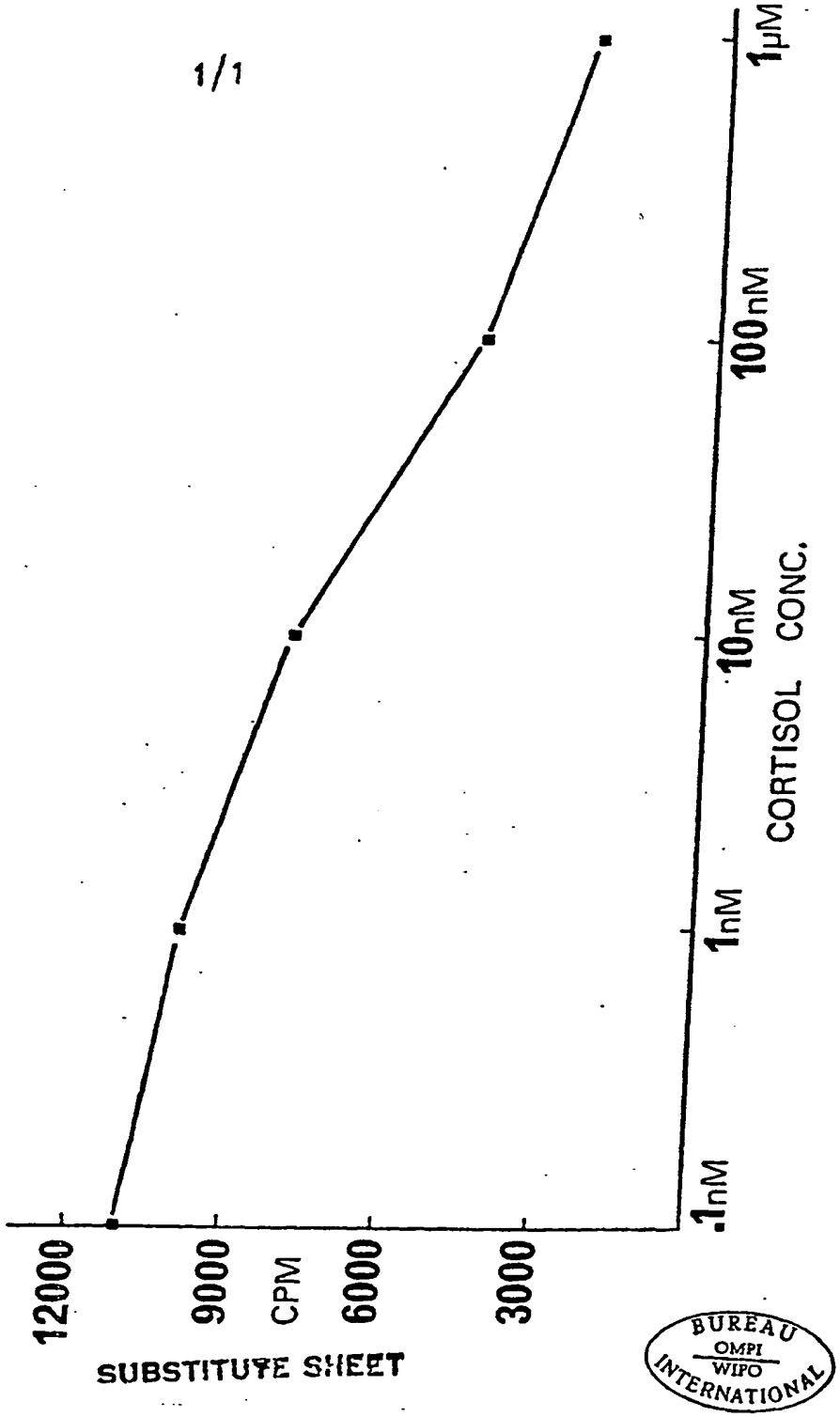

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 83/00210

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[3]: G 01 N 33/54; G 01 N 33/74

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC[3]	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [4]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [5]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X,Y	EP, A, 0015687 (R.P. EKINS) 17 September 1980 see page 5, line 16 - page 19; claims 1-10	1-5,9
Y	WO, A, 8201773 (CELLTECH LIMITED) 27 May 1982 see page 2, lines 18-32; page 4, line 33 - page 6, line 16	1,2,5,9
Y	EP, A, 0026103 (THE RADIOCHEMICAL CENTRE LTD.) 1 April 1981 see page 6, line 17 - page 9, line 5; page 10, line 9 - page 14, line 5; example 7; claims 1,4,5	1-3,5,9
Y	GB, A, 2085160 (CORNING GLASS WORKS) 21 April 1982 see the entire document	1-3,5

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [3]	Date of Mailing of this International Search Report [3]
8th December 1983	04 JAN. 1984
International Searching Authority [3]	Signature of Authorized Officer [20]
EUROPEAN PATENT OFFICE	G.L.M. Kruydenberg

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 83/00210 (SA 5719)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/12/83

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0015687	17/09/80	JP-A-	55146043	14/11/80
		US-A-	4381291	26/04/83
WO-A- 8201773	27/05/82	WO-A-	8102899	15/10/81
		EP-A-	0050129	28/04/82
		GB-A-	2083836	31/03/82
		EP-A-	0064063	10/11/82
		AU-A-	7035781	26/10/81
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		AU-B-	528427	28/04/83
GB-A- 2085160	21/04/82	FR-A-	2490826	26/03/82
		JP-A-	57086052	28/05/82
		DE-A-	3136579	19/08/82
		US-A-	4410633	18/10/83

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